

## ISOLATION AND SOME CHARACTERIZATIONS OF A GLYCOSYLATED FIBRINOLYTIC ENZYME OF EARTHWORM, *EISENIA FETIDA*

Li Li, Jing Zhao and Rong-Qiao He\*

Lab of Visual Information Processing, Center for Brain and Cognitive Sciences, Institute of Biophysics,  
Chinese Academy of Sciences, Chaoyang Dist., 15 Da-Tun Rd, Beijing 100101, China

\*Corresponding author. Tel.: +86-10-64889876; Fax: +86-10-64877837; E-mail: herq@sun5.ibp.ac.cn

**Abstract:** Resin coupled with *m*-aminophenylboronic acid was used to isolate a glycosylated component from homogenate of earthworm (*Eisenia fetida*). The fraction showed a single band on SDS-PAGE with a molecular weight of 34,193 Da determined by mass spectroscopy. The N-terminal region is AQVCCPDI, different from those of earthworm fibrinolytic enzymes reported previously (Nakajima *et al.* 1993). This glycosylated component showed an activity on digesting both Chromozym-TH and fibrin, suggesting that it is a novel fibrinolytic enzyme.

**Key Words:** Earthworm fibrinolytic enzyme; Glycosylated earthworm fibrinolytic enzyme; Isolation; Chromozym-TH; Affinity chromatography.

### INTRODUCTION

Earthworm fibrinolytic enzymes (EFEs) are a complex mixture of serine proteases [1], the purified components of which are potentially useful in research on new drugs for cerebro- and cardio-vascular thrombus. Ryu *et al.* [2,3] immobilized EFE to polyurethane using maleic anhydride methylvinyl ether copolymer (MAMEC) as an enzyme carrier. The EFE-immobilized polyurethane surface was highly antithrombogenic and had potential for reducing surface induced thrombus. And these enzymes have significant fibrinolytic effect on I<sup>125</sup>-labeled fibrin clots in blood vessels of rabbit pulmonary embolism by oral-administration [4]. Six isomers (EFE-I-0, -I-1, -I-2, -II, -III-1 and III-2), with different fibrinolytic activities, were isolated from the enzyme mixture from earthworm (*Lumbricus rubellus*) [1,5]. Mihara *et al.* [6] used a fibrin plate method to assay these isozymes. After that, two rapid methods were developed and

EFEs could be assayed conveniently [7,8]. Among the six isomers, the activities of EFE-III-1 and -III-2 were detectably the highest. Meanwhile, according to Nakajima *et al.* (1993), no component sugar could be detected in the six isomers [1].

Last year, Zhao *et al.* [9] observed four glycosylated components, which functioned like EFE, with molecular masses from 22 to 34 kDa, by affinity chromatography of a soybean trypsin inhibitor coupled with matrix, followed by a DEAE-cellulose column and gel electrophoresis. The component sugar was visualized by thymol/sulfuric acid. Some years ago, an affinity chromatography was developed to specifically isolate glycosylated proteins as described by Mallia *et al.* [10]. *m*-aminophenylbornic acid (APBA), coupled with Sepharose CL-6B, was used as the specific ligand to bind to *cis*-pyranose on proteins. In this paper, we employed the APBA-Sepharose CL-6B column to have isolated a glycosylated EFE (gEFE) and studied some properties of this novel protease.

## MATERIALS AND METHODS

### 2.1. Materials

1,1'-Carbonyl-diimidazole (CDI), bovine serum albumin (BSA), standard protein markers\_fibrinogen, thrombin and *m*-aminophenylbornic acid (APBA) were from Sigma. Goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) was from Jackson, Inc. Nitrocellulose membrane was from Life-Technologies. Chromozym TH came from Roche and Sepharose CL-6B was from Pharmacia. Other reagents used were analytic grade without further purifying. We raised antiserum against EFE-II by immunizing New Zealand white male rabbits as described before [11].

### 2.2. Preparation of APBA-Matrix

The matrix was activated by CDI according to Bethell *et al.* [12], before the ligand APBA was added to couple with resin. APBA (0.2 g) was dissolved in 0.2 M NaHCO<sub>3</sub> buffer (pH 8.5, 10 mL) containing 0.15 M NaCl at room temperature, mixed with the activated matrix (10 mL) and slightly shaken for 0.5 h [10].

### 2.3. Purification

Crude EFEs (50 mg), prepared as described Zhou [5], obtained from saline extract of earthworm *Eisenia fetida*, were solubilized in 0.05 M Tris-HCl buffer (pH 7.2, 2 mL) and applied on the APBA-Sepharose CL-6B column (1.6×20 cm) [13] after the column was equilibrated with 0.2 M asparagine buffer (pH 9.15), containing 0.02 M magnesium chloride at 4°C. 0.02 % sodium azide was added as an antimicrobial agent to each buffer. 0.001 M Tris-HCl buffer (pH 9.18), containing 0.002 M asparagine and 0.1 M magnesium chloride, was used as the mobile phase. After that, the glycosylated component was eluted off the matrix with 0.2 M *D*-sorbitol in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.005 M asparagine. The eluent was pooled and dialyzed against 0.01 M Tris-HCl buffer (pH 7.2). Then gEFE was lyophilized and stored at -20°C until use. The concentration of gEFE was estimated as described by Bradford [14].

#### 2.4. SDS-PAGE

We electrophoresed gEFE (10  $\mu$ g) with 15% SDS-PAGE (100V) and visualized the protein bands by Coomassie Brilliant Blue R-250.

#### 2.5. N-terminal sequencing

The protein bands in the gel were transferred onto PVDF membrane, and then the N-terminal region was sequenced by Edman degradation on a Shimadzu PPSQ-10 Automated Protein Sequencer.

#### 2.6. Mass Spectrometry (MS)

MALDI mass spectrum of the glycosylated component was performed on a Time-of-Flight (TOF) mass spectrometer (REFLEX III, Bruker Inc.) equipped with a model VSL-337ND nitrogen Laser (LASER Sci.) according to Escoubas *et al.* [15]. All experiments were performed using the saturated  $\alpha$ -cyano-4-hydroxycinnamic acid (CCA), dissolved in 50% (v/v) solution of acetonitrile and aqueous 0.2% TFA, as the matrix. This saturated solution was mixed with 0.1 mg/mL peptide samples (1  $\mu$ L) at a ratio of 4:1 (v/v), applied to the stainless steel sample plate and vacuum-dried before being measured in the mass spectrometer.

#### 2.7. Western blotting

Samples in the SDS-PAGE were transferred onto nitrocellulose membrane, which was used for immunoblotting referred to Sambrook *et al.* [16]. We added rabbit anti-EFE-II (1:500), and goat anti-rabbit IgG conjugated with HRP (1:5000) as the secondary antibody. The membrane was visualized with DAB in PBS containing 0.015 % hydrogen peroxide. And then it was stained by Ponceau S Red to exhibit all the protein bands on it. EFE-II was used as control.

#### 2.8. Enzymatic kinetic assays

The enzymatic assays including  $K_m$  and  $V_m$  of gEFE and EFE-II were measured with chromogenic substrate as described previously [7]: EFE (final conc. 1  $\mu$ M) dissolved in Tris-HCl buffer (pH 7.2) was incubated at 25°C for 3 min before Chromozym TH (Tos-Gly-Pro-Arg-4-NA, final conc. 60-450  $\mu$ M) was added. During the assay, the substrate was cleaved and produced a chromogenic compound, 4-nitraniline ( $\epsilon_{405\text{ nm}} = 9.75\text{ mM}^{-1} \times \text{cm}^{-1}$ ). The change of absorbance at 405 nm was recorded on a spectrophotometer (PE-12). The optimal pH and temperature were measured using the same substrate. The buffers, 0.05M  $\text{Na}_2\text{HPO}_4$ -citrate (pH 5.0-7.0); Tris-HCl (pH 8.0-8.9) and Gly-NaOH (pH 9.5-12.1), were used to determine the optimal reaction pH. gEFE was incubated in these buffers at 25°C for 10 min before 0.15mM chromozym-TH was added. To measure the activity at different temperatures (18, 25, 37, 45, 55, 65 and 75°C), 0.05M Tris-HCl (pH 7.2) was carried out under the same condition.

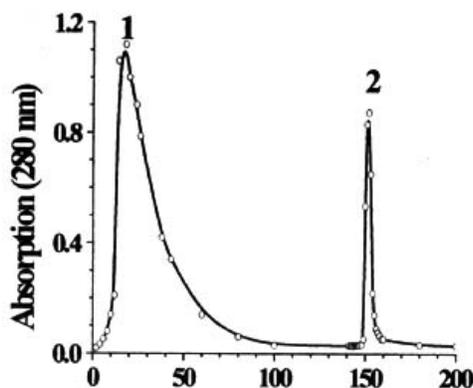
### 2.9. Fibrinolytic assay

As described by Zhou *et al.* [8], Rayleigh-Light Scattering was used to assay gEFE and EFE-II (0.1  $\mu$ M as final conc.) on a fluorescence spectrophotometer (Hitachi F-4500), respectively. The enzyme was incubated with fibrinogen (60  $\mu$ g/ml, as final conc.) in 0.05 M Tris-HCl buffer (pH 7.2) for 10 min, thrombin (6  $\mu$ g/ml) was added into the mixture, and the reaction was followed by the measurement of the light scattering at 480 nm.

## RESULTS AND DISCUSSION

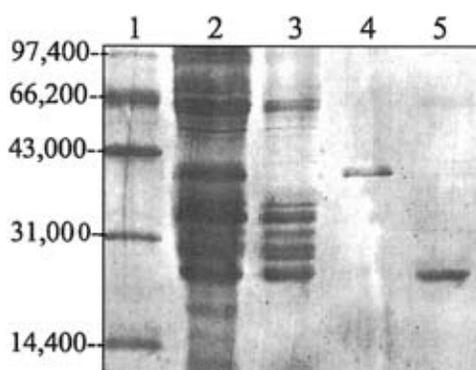
### 3.1. Purification

As shown in Figure 1, affinity chromatography (APBA-Sepharose CL-6B column) of crude EFEs resulted in two absorption peaks, the flow-through and the glycosylated component eluted specifically by sorbitol. The absorbance at 280 nm resulted from the eluted protein, because the reference used in the spectrophotometer was the elution buffer, containing the equivalent sorbitol. The gEFE was approx. 6 percent of the crude enzymes, determined by the method of Bradford [14]. The glycosylated component

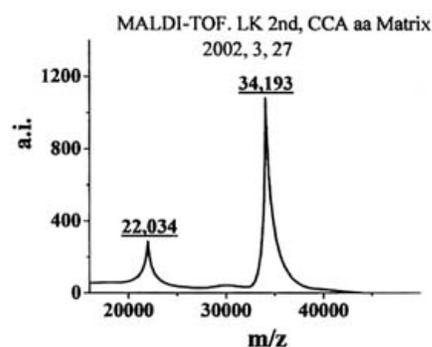


**Figure 1. Elution profile of affinity chromatography.** Crude earthworm fibrinolytic enzymes (50 mg) were applied on an APBA-Sepharose CL-6B column (1.6 $\times$ 20 cm). The first 120 mL of elution buffer was 0.001 M Tris-HCl buffer (pH 9.18), containing 0.002 M asparagine and 0.1 M MgCl<sub>2</sub>, while the following 80 mL of elution buffer was 0.2 M *D*-sorbitol in 0.1 M Tris-HCl buffer (pH 7.2). The ordinate was the absorbance (280 nm) of protein in fractions. Peaks 1 and 2 represent flow-through and specific eluent, respectively.

showed a single band in SDS-PAGE, with an apparent molecular mass  $\sim$  40 kDa (Figure 2). On the other hand, there was no major band at the same position in the unbound (flow-through) fraction. The component sugar of the glycosylated enzyme should be a *cis*-pyranose, which could be recognized by *m*-APBA-Sepharose column. Result of mass spectrometry showed that molecular mass of gEFE was 34,193 Da (Figure 3), different from that estimated by the electrophoresis. It was reported that glycoprotein



**Figure 2. 15% SDS-PAGE of the glycosylated protein.** Lane 1: standard protein markers; Lane 2: the crude EFes (10 µg); Lane 3: elution from unspecific pool; lane 4: elution from specific pool and lane 5: EFE-II (1 µg) as control.



**Figure 3. MALDI-TOF mass spectrum of glycosylated component.** gEFE (10 µg) was analyzed on a Reflex III time-of-flight (TOF) mass spectrometer equipped with a model VSL-337ND nitrogen Laser according to Escoubas et al [15]. Saturated CCA in solution of acetonitrile (50%, v/v) and TFA (0.2%, v/v) was used as the matrix.

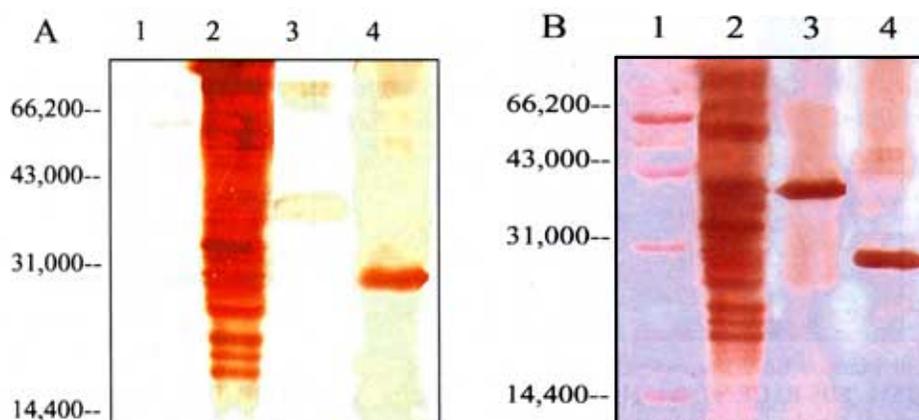
exhibited an anomalously low free electrophoretic mobility compared with those of non-glycosylated proteins [17]. It suggested that the retardation of gEFE on SDS-PAGE was due to the sugar contents bound to the enzyme.

### 3.2. Structure of N-terminal region

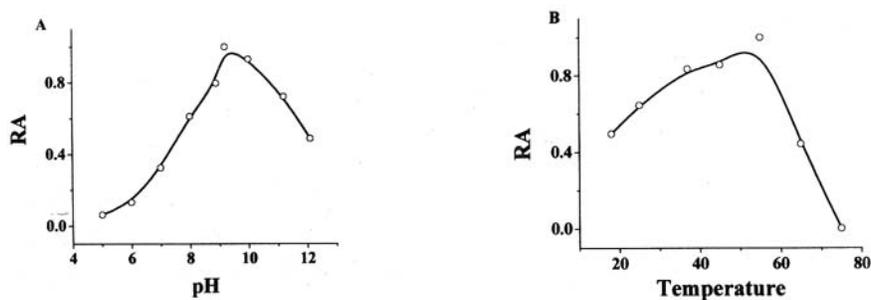
Sequencing of the N-terminal region (the first 8 amino acid residues) indicated again that gEFE was a highly purified component. The N-terminal sequence, A-Q-V-C-C-P-D-I-, showed no similarities with the isomers of EFes and other trypsin-like serine proteases, reported previously. It was different from the N-terminal sequences of the glycosylated isozymes described by Ru *et al.* [18]. For earthworm proteases, different methods could isolate different isozymes. This was confirmed by western blot: the band of gEFE was not specifically recognized by rabbit anti-EFE-II, as shown in Figure 4. The homology of the N-terminal region has been investigated by a computer search of *Non-Redundant Protein Sequence Data Bank*, also suggesting no similarities with serine proteases. Nevertheless, the N-terminal eight residues could not be enough to classify this enzyme. Whether the sequence downstream is homologous to those of serine proteases needs further studying.

### 3.3. Some properties of gEFE

The  $K_m$  of gEFE with Chromozym TH as a substrate was 30.65 µM and the  $V_m$  was 0.044 µM·s<sup>-1</sup> (non-glycosylated, for instance, EFE-II,  $K_m$  and  $V_m$  were 6.4 µM and 0.9 µM·s<sup>-1</sup>, respectively). The optimal pH range for gEFE activity was between 8.0 and 11.2 (Figure 5A), so we selected pH 8.2 for the thermal assay.



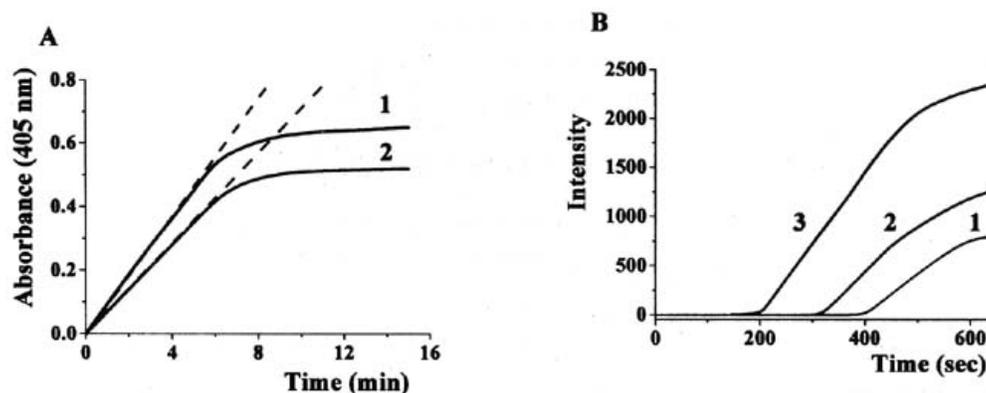
**Figure 4. Western blotting of glycosylated fibrinolytic enzyme.** Lane 1: standard protein markers; Lane 2: crude EFEs at 100 µg/mL; Lane 3: elution from specific pool; Lane 4: EFE-II at 10 µg/mL elution. A. The membrane was incubated with rabbit anti-EFE-II (1:500), and goat anti-rabbit IgG conjugated with HRP (1:5000) as the secondary antibody before visualized with DAB in PBS containing 0.015 % hydrogen peroxide. B. Staining by Ponceau S Red.



**Figure 5. The optimal pH and Temperature.** A. Effect of pH on the activity of gEFE, Chromozym TH as substrate, was measured in 0.05M Tris-HCl, pH 7.2 containing 227 mM NaCl. The buffers with various pH used were 0.05 M Na<sub>2</sub>HPO<sub>4</sub>-citrate (pH 5.0-7.0), Tris-HCl (pH 8.0-8.9) and Gly-NaOH (pH 9.5-12). B. The thermal-stabilized test of gEFE was measured at different temperatures (18, 25, 37, 45, 55, 65 and 75°C, respectively).

Although the activity reached the maximum at 55°C (Figure 5B), we chose 25°C as the detection temperature because it is easy to control the experiment at this temperature. In addition, it suggests the gEFE may be a heat-stable enzyme.

Although the N-terminal sequence of gEFE manifested no similarities to those of serine proteases, it is necessary to investigate whether the glycosylated enzyme could react with the substrates for serine



**Figure 6. Assay of gEFE and comparison with EFE-II.** Curve 1: activity of EFE-II; Curve 2: that of gEFE. A. Chromozym TH (final conc. 50  $\mu$ M) was added to the enzyme (final conc. 1  $\mu$ M) dissolved in Tris-HCl buffer (pH 7.2) before the OD<sub>405</sub> was recorded. B. The enzyme (final conc. 1  $\mu$ M) was incubated with fibrinogen (final conc. 60  $\mu$ g/ml) in Tris-HCl buffer (pH 7.2) for 10 min before thrombin (final conc. 6  $\mu$ g/ml) was added. The light scattering was measured at 480 nm. The mixture of fibrinogen and thrombin at the same concentration was used as control (Curve 3).

proteases. First, we utilized Chromozym TH to assay gEFE. The glycosylated enzyme was highly active (approx. 80% of EFE-II) when reacted with the specific chromogenic substrate for thrombin as shown in Figure 6A. Among known EFEs, EFE-II has a high specific activity: 48 units/mg (one unit was defined as the amount of enzyme causing transformation of 1  $\mu$ mol of substrate per minute at 25°C under optimal conditions of measurement). At the same time, when we used fibrinogen as substrate in the presence of thrombin as reported previously [5], gEFE's fibrinolytic activity was about 60% compared to that of EFE-II determined by monitoring light scattering assay at 480 nm at room temperature (Figure 6B). It appears that the glycosylated enzyme is a new kind of serine proteases, with a fibrinolytic activity.

#### ACKNOWLEDGEMENTS

We are grateful to Mr. Yia-Jie Xu for his kind supporting and helpful suggestions.

#### REFERENCES

- [1] Nakajima, N., Mihara, H., Sumi, N. (1993) *Biosci. Biotech. Biochem.* 57, 1726-1730.
- [2] Ryu, G.H., Park, S., Kim, M., Han, D.K., Kim, Y.H., Min, B. (1994) *J. Biomed. Mater. Res.* 28, 1069-1077.
- [3] Park, Y., Ryu, E., Kim, H., Jeong, J., Kim, J., Shim, J., Jeon, S., Jo, Y., Kim, W., Min, B. (1999) *Artif. Organs* 23, 210-214.
- [4] Shun, Y.P., Ging, Y.Y., Fan, R., Wu, C. (1998) *Capital Medicine Sinica.* 5, 17-18.
- [5] Zhou, J., Wu, C., He, R.Q. (1998) *J. Biochem. Mol. Bio. & Biophys.* 5, 1-5.
- [6] Mihara, H., Sumi, H., Mizumoto, H., Ikeda, R., Seiki, M., Naruyama, M. (1991) *J. Physiol.* 41, 461-472.
- [7] Zhou, J., Fan, R., Wu, C., He, R.Q. (1997) *Protein and Peptide letters*, 4, 409-414.

- [8] Zhou, J., Jiang, K., He, R.Q., Han, Y.M. (1997) *Acta Biophysica Sinica*. 13, 531-535.
- [9] Zhao, X.Y., Jing, T.Y. (2001) *Prog. Biochem. Biophys.* 28, 218-221.
- [10] Mallia, A.K., Hermanson, G.T., Krohn, R.I., Fujimoto, E.K., Smith, P.K. (1981) *Analytical Letter* 14, 649-661.
- [11] Fan, Q., Wu, C., Li, L., Fan, R., Wu, C., Hou, Q.M., He, R.Q. (2001) *Biochim. Biophys. Acta* 1526, 286-292.
- [12] Bethell, G.S., Ayers, J.S., Hancock, W.S. (1979) *J. Bio. Chem.* 254, 2572-2574.
- [13] He, R.Q., Yang, M.D., Zheng, X., Zhou, J.X. (1995) *Biochem. J.* 309, 133-139.
- [14] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.
- [15] Escoubas, P., Corzo, G., Whiteley, B.J., Celerier, M.L., Nakajima, T. (2002) *Rapid Commun. Mass Spectrom.*, 16, 403-413.
- [16] Sambrook, J.E., Fritsch, F., Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 18.61-18.70.
- [17] Marciani, D.J., Papamatheakis, J.D. (1978) *J. Virol.* 26, 825-827.
- [18] Xie, J.B., Guo, Z.Q., Weng, N., Wang, H.T., Jiang G.Q., Ru B.G. (2003) *Prog. Biochem. Biophys.* 30, in press.

Received on April 18, 2002, accepted on January 9, 2003.